FEBS Letters 564 (2004) 58–62 FEBS 28266

# Characterizing the interaction of the mammalian eIF4E-related protein 4EHP with 4E-BP1

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Received 11 February 2004; accepted 2 March 2004

First published online 25 March 2004

Edited by Lev Kisselev

**Abstract Eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) represses translation initiation by binding to eukaryotic initiation factor 4E (eIF4E). 4E-BP1 also binds to the eIF4E homologous protein (4EHP). We show that eIF4E-binding mutants of 4E-BP1 (Y54A and L59A) fail to form heterodimeric complexes with wild-type 4EHP. In addition, the W95A mutant of 4EHP, similar to a homologous mutation in eIF4E, inhibits its binding to wild-type 4E-BP1. Interestingly, 4EHP over-expression instigates a negative feedback loop that inhibits upstream signaling to 4E-BP1 and ribosomal protein S6 kinase 1 (S6K1) whereas the 4E-BP1-binding-deficient mutant of 4EHP(W95A) was unable to trigger this feedback loop. Thus, the interaction of 4EHP with 4E-BP1 is necessary for this observed impaired signaling to 4E-BP1 and S6K1.**


*Key words:* Eukaryotic initiation factor 4E-binding protein 1; Eukaryotic initiation factor 4E homologous protein; Eukaryotic initiation factor 4E; Translation; Initiation factor; 7-Methylguanosine triphosphate

## 1. Introduction

Protein synthesis requires three stages of mRNA translation termed initiation, elongation, and termination. Control of protein synthesis is largely governed by a cohort of eukaryotic initiation factors (eIFs) that mediate specific steps in the initiation process (for review see [1]). A rate-limiting step in translation initiation involves the formation of the eIF4F complex that recruits ribosomal subunits to the mRNA, a process known as cap-dependent translation. The cap-binding pocket of eIF4E interacts with the cap moiety (7-methylguanosine triphosphate ($m^7$GTP)) positioned at the extreme 5′ end of the mRNA. eIF4E interacts with eIF4G that in turn binds to eIF4A, a bi-directional RNA helicase, to form the eIF4F complex. eIF4G is a large scaffold protein that recruits many other factors to the 5′ cap structure, including poly(A)-binding protein, eIF3, and the eIF4E kinases, Mnk1 and Mnk2 (see review [1]). Formation of the eIF4F complex, and thus cap-dependent translation, is antagonized by eIF4E-binding proteins (4E-BPs), during conditions of low mitogenic and nutrition sufficiency.

The eIF4E-binding motif of 4E-BP1 interacts with a region of eIF4E, which also binds to eIF4G [2]. Therefore, interaction of 4E-BP1 to eIF4E blocks eIF4F complex formation by preventing the binding of eIF4G to eIF4E. Phosphorylation of 4E-BP1 antagonizes its binding to eIF4E. During conditions of mitogenic and nutrient sufficiency, 4E-BP1 becomes phosphorylated and dissociates from eIF4E. This allows eIF4E to participate in the formation of the eIF4F complex. 4E-BP1 phosphorylation occurs in a hierarchical manner at seven characterized (Ser/Thr)Pro-directed sites (Thr37, -46, and -70 and Ser65 -82, -101, and -112, numbering according to the human sequence) [3,4]. Phosphorylation of sites that lie proximal to the eIF4E-binding domain of 4E-BP1, Thr46 and Ser65, is considered to be critical for disrupting the binding of 4E-BP1 to eIF4E.

Given that the regulation of 4E-BP1 is complex, it is likely that 4E-BP1 interacts with many signaling components that are presently uncharacterized. To identify potential 4E-BP1-interacting proteins we carried out a yeast two-hybrid screen using 4E-BP1 as bait. We discovered that eIF4E homologous protein (4EHP) interacted with the eIF4E-binding motif of 4E-BP1. In this study, we further characterize this interaction and discover that high levels of 4EHP protein can trigger a negative feedback loop in cells that inhibits upstream signaling towards both 4E-BP1 and ribosomal protein S6 kinase 1 (S6K1).

## 2. Materials and methods

### 2.1. Plasmids

Mouse 4E-BP1 (a gift from J. Lawrence, University of Virginia School of Medicine, Virginia) was subcloned into pGBDU-C3 (provided by S.M. Hollenberg, Vollum Institute, Oregon) for use in the yeast two-hybrid screen. pACTAG2 expressing human HA-tagged 4E-BP1 was provided by N. Sonenberg (McGill University, Canada). Human 4EHP (ATCC number 7387103) and eIF4E (provided by N. Sonenberg) were subcloned into pRK7 so that they were expressed with an N-terminal Flag epitope (MDYDDDDK). S6K1 and F5A-Δct S6K1 expression vectors were generated as previously described [5]. Point mutants were generated by site-directed mutagenesis (QuikChange; Stratagene).

### 2.2. Analysis of protein phosphorylation and association of translation factors

Human embryonic kidney 293E (HEK293E) cells were cultured (at 37°C within 5% $CO_2$) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. $CaPo_4$ transfec-

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*Abbreviations:* eIF4E, eukaryotic initiation factor 4E; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; 4EHP, eIF4E homologous protein; HEK293E, human embryonic kidney 293E; S6K1, ribosomal protein S6 kinase 1; $m^7$GTP, 7-methylguanosine triphosphate

0014-5793/04/$30.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(04)00313-8

tions were performed as previously described [5]. After treatment, cells were harvested and Western blot analysis was carried out as before [6]. Anti-Flag antibodies (M2) were purchased (Eastman Kodak, Newhaven, CT, USA). Anti-HA antibody was a kindly provided by M. Chou (University of Pennsylvania) and anti-4E-BP1 phospho-specific antibodies were bought from Cell Signaling Technology. eIF4E and 4EHP was purified by using affinity chromatography on $m^7$GTP-Sepharose as previously described [7]. Cross-linking reaction was carried out with DSP (Pierce) as previously described [8].

### 2.3. Immunoprecipitation and immune complex kinase assays

For immunoprecipitation studies of HA-tagged S6K1, cell extracts were immunoprecipitated with an anti-HA antibody bound to protein A-Sepharose (Pharmacia) for 3 h. Immunoprecipitates were washed as previously described [9]. Kinase activity of S6K1 was then determined using recombinant glutathione *S*-transferase (GST)-S6 as a substrate, as previously described [9].

## 3. Results

### 3.1. 4EHP interacts with the eIF4E-binding domain of 4E-BP1

Yeast two-hybrid analysis was carried out to identify novel interactors of 4E-BP1. A PJ69-4A yeast strain was first transformed with pGBDU-C3, which contained full-length mouse 4E-BP1 fused to the Gal4 DNA-binding domain. The PJ69-4A yeast strain containing 4E-BP1 in pGBDU-C3 was then transformed with a two-hybrid cDNA library created from day 9.5 and 10.5 mouse embryos [10]. Approximately $2\times10^7$ transformants were screened, which is 100 times the complexity of the library after amplification. Fifty-one His+ colonies were obtained from the primary screen, of which 29 were further selected with greater stringency in the absence of adenine. The library plasmids were then recovered and sequenced. Nine of the isolated plasmids contained sequences encoding all or part of 4EHP, which is highly homologous to eIF4E (30% identical and 60% similar, see Fig. 1A). eIF4E, a well characterized translation initiation factor that interacts with 4E-BP1, was also recovered from the screen (see review [1]).

To examine whether 4EHP bound to 4E-BP1 in a manner analogous to that of eIF4E, 4E-BP1 mutants with single (Y54A and L59A) and double (Y54A/L59A) point mutations within the eIF4E-binding domain of 4E-BP1 were generated. Both single and double point mutants of 4E-BP1 abolished 4EHP's interaction with 4E-BP1, indicating that 4EHP binds to the eIF4E-binding domain of 4E-BP1 (Fig. 1B). Binding of 4EHP to 4E-BP1 is likely weaker than that of eIF4E to 4E-BP1 as eIF4E was still able to interact with the single point mutants of 4E-BP1 (Y54A and L59A) while 4EHP was not.

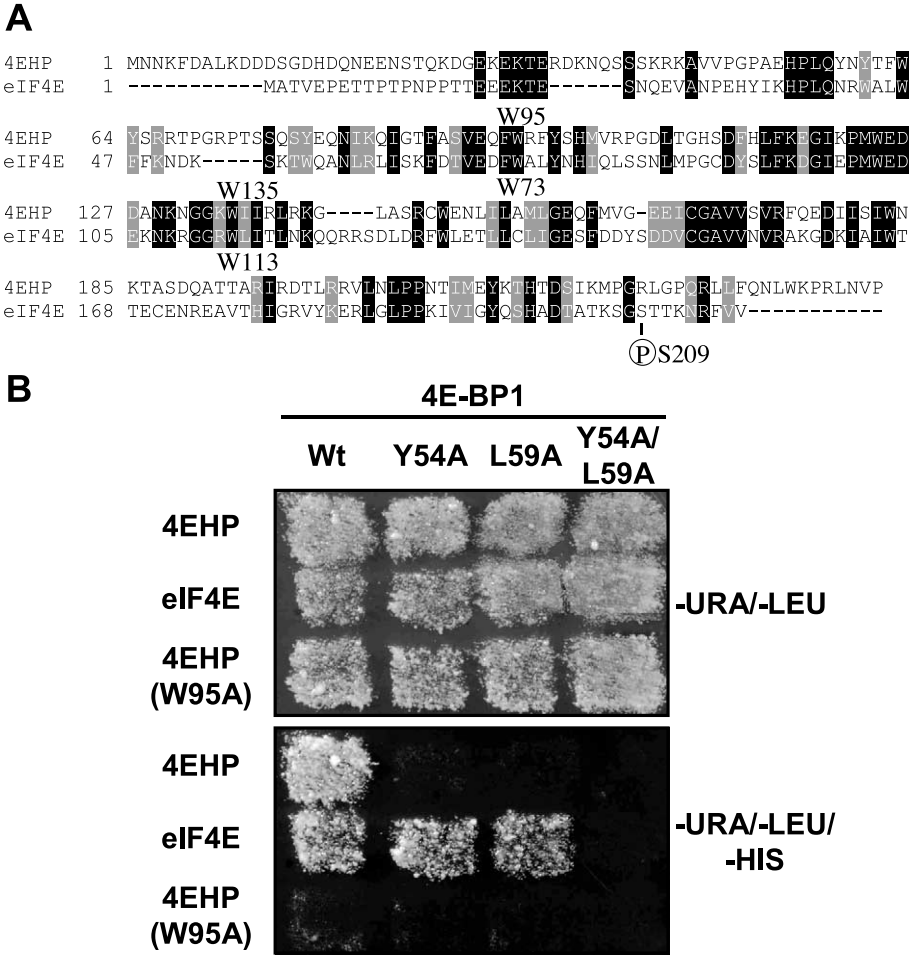


Fig. 1. 4E-BP1 interacts with 4EHP through the eIF4E-binding motif. A: Amino acid alignment of human 4EHP and eIF4E. Conserved Trp residues are labeled accordingly. The Ser209 phosphorylation site within eIF4E is also marked. B: PJ69-4A yeast transformed with DNA-binding fusion proteins (4E-BP1 wild-type (Wt), and 4E-BP1 (Y54A, L59A, and Y54A/L59A)) and activation domain fusion proteins (4EHP, eIF4E, and 4EHP(W95A)) were grown on media lacking uracil (−URA) and leucine (−LEU). Positive interaction was determined by growth on minus histidine plates (−HIS).

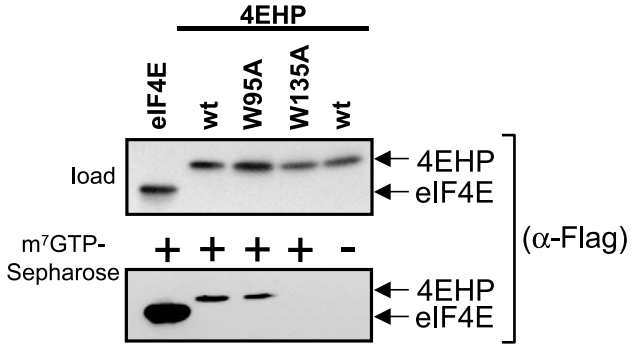


Fig. 2. 4EHP(W95A) still retains m$^7$GTP affinity. Flag-tagged eIF4E, 4EHP, 4EHP(W95A) and 4EHP(W115A) were over-expressed in HEK293E cells and purified on m$^7$GTP-Sepharose beads from cell lysates (bottom panel). The amount of protein expressed is shown in the upper panel ('load').

It is known that Trp73 within eIF4E is required for eIF4E's interaction with 4E-BP1 [11,12]. Based on the sequence alignment (Trp95 of 4EHP aligns with Trp73 of eIF4E, Fig. 1A), we generated a mutant of 4EHP where Trp95 was substituted to alanine. Given that Trp73 lies on the dorsal surface of eIF4E [12], it is likely that the Trp95 residue of 4EHP is also exposed and interacts with 4E-BP1. Indeed, the 4EHP-(W95A) mutant was unable to associate with 4E-BP1 (Fig. 1B), revealing that a homologous region within both eIF4E and 4EHP is required for 4E-BP1 binding.

### 3.2. The 4EHP(W95A) but not the 4EHP(W135A) mutant associates with the m$^7$GTP cap moiety

Previously, 4EHP was shown to interact with the m$^7$GTP cap moiety that is located at the extreme 5′ end of most mammalian mRNA transcripts [13]. These studies also revealed that a mutant of 4EHP with Trp135 substituted to an alanine prevented cap binding [13]. Trp135 of 4EHP aligns with Trp113 of eIF4E (see Fig. 1A), a residue that is required for the binding of eIF4E to the m$^7$GTP cap structure [13]. Purification of wild-type and mutant forms of 4EHP on m$^7$GTP-Sepharose was compared to that of eIF4E (Fig. 2). Both eIF4E and 4EHP associated with the m$^7$GTP cap structure. eIF4E, however, was more abundantly purified than 4EHP using this technique even though approximately equal amounts of starting material were used. The 4EHP(W95A) mutant protein, which was unable to interact with 4E-BP1 (Fig. 1B), was also purified using m$^7$GTP affinity chromatography while the 4EHP(W135A) mutant was not (Fig. 2). Therefore, mutation of Trp95 to alanine is sufficient to block the interaction of 4E-BP1 with 4EHP (Fig. 1B) but does not prevent the binding of 4EHP with the m$^7$GTP cap structure (Fig. 2).

### 3.3. 4EHP over-expression impairs insulin-stimulated 4E-BP1 phosphorylation, which requires 4E-BP1's interaction with 4EHP

Previously it was shown that 4EHP did not co-purify with 4E-BP1 using standard immunoprecipitation methods [13]. Similarly, we were unable to co-purify 4E-BP1 with 4EHP

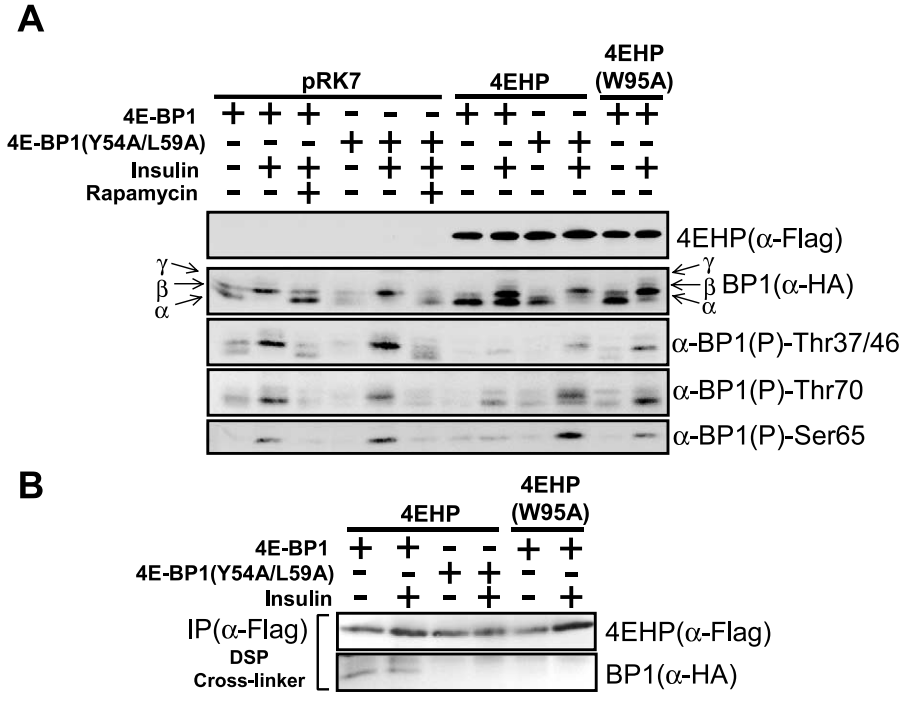


Fig. 3. 4EHP over-expression impairs insulin-stimulated 4E-BP1 phosphorylation and requires 4E-BP1 interaction with 4EHP. A: HEK293E cells co-expressing Flag-tagged 4EHP and 4EHP(W95A) with either HA-tagged wild-type 4E-BP1 or 4E-BP1(Y54A/L59A) were serum-starved. These cells were pre-treated with 20 nM rapamycin (rap) for 30 min prior to being stimulated with insulin (100 nM) for 30 min as indicated. The amount of 4EHP expressed was determined with the anti-Flag antibody. Phosphorylation of ectopically expressed 4E-BP1 was determined with anti-HA and phospho-specific 4E-BP1 antibodies that recognize pThr37 and/or pThr46, pSer65, or pThr70, as indicated. The α-, β-, and γ-species of 4E-BP1 are indicated. B: Cells treated as in A were lysed in the presence of DSP cross-linker as described in Section 2. Flag-tagged 4EHP was immunoprecipitated with the anti-Flag antibody and the amount of HA-tagged 4E-BP1 associated with 4EHP was determined.

using routine immunoprecipitation procedures (data not shown). Interestingly, during these preliminary experiments we observed that over-expression of 4EHP impaired 4E-BP1 phosphorylation (data not shown).

To extend this finding further, N-terminal Flag-tagged wild-type 4EHP or 4EHP(W95A) were over-expressed in serum-starved HEK293E cells and the phosphorylation of co-expressed N-terminal HA-tagged wild-type 4E-BP1 or 4E-BP1-(Y54A/L59A) was investigated after the cells were stimulated with insulin (Fig. 3A). Three different phosphorylated species of 4E-BP1 resolve on sodium dodecyl sulfate–polyacrylamide gel electrophoresis with γ- and α-isoforms being the most and least phosphorylated species, respectively. Over-expression of wild-type 4EHP blocked the basal and the insulin-stimulated phosphorylation of wild-type 4E-BP1 as observed by a higher proportion of 4E-BP1 that resolved as the least phosphorylated α-species, when compared to the pRK7 vector only control. Over-expression of wild-type 4EHP also repressed the phosphorylation of 4E-BP1 at Thr37 and/or Thr46, Ser65, and Thr70. Interestingly, wild-type 4EHP did not inhibit the phosphorylation of 4E-BP1(Y54A/L59A), a mutant of 4E-BP1 that cannot interact with either eIF4E or 4EHP (see Fig. 1B). Similarly, over-expression of 4EHP- (W95A), a mutant of 4EHP that cannot associate with 4E-BP1 (see Fig. 1B), did not impair wild-type 4E-BP1 phosphorylation. These data indicate that 4EHP must interact with 4E-BP1 to impair its phosphorylation.

The above data imply that 4EHP interacts with 4E-BP1 within cells. To investigate this interaction in more detail, we prepared cell extracts in the presence of a DSP cross-linker to maintain the initiation complexes (Fig. 3B). Wild-type 4E-BP1 associated with wild-type 4EHP while no interaction was observed when binding mutants of either 4E-BP1 or 4EHP were used. Insulin stimulation is known to cause the dissociation of 4E-BP1 from eIF4E as a result of increased 4E-BP1 phosphorylation [3,4]. Insulin did not induce the release of 4E-BP1 from 4EHP (Fig. 3B) and can be explained by the impaired phosphorylation state of 4E-BP1, when 4EHP is expressed to high levels (see Fig. 3A).

### 3.4. *High expression of wild-type 4EHP but not the W95A mutant inhibits S6K1 activation*

High levels of eIF4E expression have also been shown to trigger a negative signaling feedback loop leading to the de-

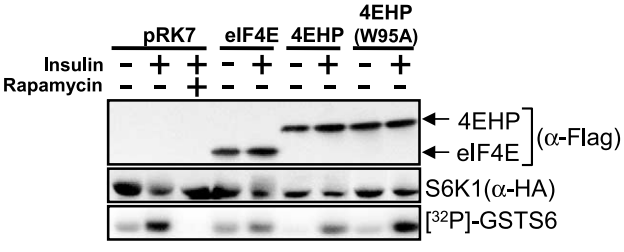


Fig. 4. Over-expression of 4EHP but not 4EHP(W95A) impairs S6K1 activation. HEK293E cells co-expressing HA-tagged S6K1 with either Flag-tagged eIF4E, 4EHP or 4EHP(W95A) were serum-starved. The cells were pre-treated with 25 nM rapamycin for 30 min and then stimulated with 100 nM insulin where indicated. Protein levels of eIF4E, 4EHP and S6K1 were determined. S6K1 activity assays were carried out as described in Section 2. Incorporation of $^{32}P$ label into GST-S6 substrate is shown in the bottom panel. An autoradiograph of the gel is presented.

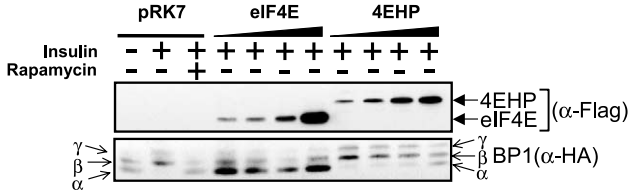


Fig. 5. eIF4E is more potent at impairing insulin-stimulated 4E-BP1 phosphorylation than 4EHP. Increasing amounts of either eIF4E or 4EHP were co-expressed with HA-tagged 4E-BP1 in serum-starved HEK293E that were stimulated with 100 nM insulin for 30 min, where indicated. The levels of eIF4E and 4EHP protein and the extent of 4E-BP1 phosphorylation were determined.

phosphorylation of 4E-BP1 and S6K1 [14]. Therefore, we wanted to determine whether 4EHP over-expression could similarly impair insulin-induced S6K1 activation. Both wild-type eIF4E and 4EHP over-expression significantly impaired insulin-stimulated S6K1 activation (Fig. 4). In contrast, the mutant of 4EHP that does not interact with 4E-BP1 (4EHP-(W95A), see Figs. 1B and 3B) did not inhibit insulin-induced S6K1 activation. These data suggest that 4EHP must form a complex with 4E-BP1 to instigate this negative feedback loop that occurs upstream of both 4E-BP1 and S6K1.

### 3.5. *High expression of either eIF4E or 4EHP inhibits 4E-BP1 phosphorylation*

Our studies reveal that a negative signaling feedback loop towards both 4E-BP1 and S6K1 is activated when high levels of 4EHP protein are present within mammalian cells, which is similar to that observed when eIF4E is expressed at high levels. Given that the binding affinity of 4EHP to 4E-BP1 was weaker than that observed for eIF4E to 4E-BP1 (Fig. 1B), we wanted to determine whether eIF4E was more potent at inducing this negative feedback loop. To do this we expressed different amounts of eIF4E and 4EHP protein with a fixed amount of 4E-BP1 in HEK293E cells and monitored 4E-BP1 phosphorylation (Fig. 5). Based on this analysis, we observed a significant difference in the ability of over-expressed eIF4E and 4EHP to initiate the observed feedback loop. For instance, eIF4E prevented insulin-stimulated 4E-BP1 phosphorylation at the lowest concentrations used whereas 4EHP yielded partial inhibition of 4E-BP1 phosphorylation at the highest amount ectopically expressed in this representative experiment.

## 4. Discussion

In this study we show that 4E-BP1 interacts with 4EHP in a manner similar to that of eIF4E. We also show that 4EHP interacts with the known eIF4E-binding motif within 4E-BP1 (Figs. 1B and 3B). Furthermore, we demonstrate that Trp95 within 4EHP is a critical residue that mediates this interaction (Figs. 1B and 3B). Importantly, the 4EHP(W95A) mutant can be affinity purified with the $m^7GTP$ cap analogue (Fig. 2), showing that Trp95 residue is involved in 4E-BP1/4EHP complex formation rather than cap binding. Our findings also suggest that the binding affinity of 4EHP to 4E-BP1 is weaker than that of eIF4E with 4E-BP1. For instance, eIF4E still interacts with 4E-BP1 possessing single amino acid point mutants within the eIF4E-binding motif (either Y54A or L59A), while 4EHP did not (Fig. 1B). Indeed, routine immunoprecip-

itation methods are sufficient to maintain 4E-BP1/eIF4E complexes but not 4E-BP1/4EHP complexes implying that association of 4EHP with 4E-BP1 is disrupted upon low salt and detergent conditions (see [13]).

This work also extends the original studies that showed that high expression levels of eIF4E turned off translation initiation through impaired signaling to 4E-BP1 and S6K1 [14]. We show that this mechanism also applies to 4EHP, where a high level of 4EHP expression triggers this negative feedback loop. We show that the activation of this feedback mechanism is directly caused by the formation of either 4E-BP1/4EHP or 4E-BP1/eIF4E complexes (Figs. 1A and 5). Interestingly, higher levels of 4EHP expression were required to inhibit 4E-BP1 phosphorylation, when compared to eIF4E (Fig. 5). This observation can be explained by the stronger binding affinity that eIF4E has with 4E-BP1 when compared to 4EHP. So what is the purpose of this negative feedback loop? It is known that a high level of eIF4E expression causes transformation [15]. Therefore, it may be possible that this negative feedback loop functions as a checkpoint control mechanism that is utilized to protect cells from an inappropriately high translation rate when eIF4E or 4EHP are vastly over-expressed.

*Acknowledgements:* A post-doctoral fellowship ALT2002-463 was awarded by the European Molecular Biology Organization to A.R.T. to fund this research. We thank The Tuberous Sclerosis Alliance and The Rothberg Courage Fund. This work was supported by National Institutes of Health (NIH) Grant GM51405 and by The TSC Alliance awarded to J.B.

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